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A paclitaxel resistant cell line, KPTA5, was established by co-selecting the parental cell line K562 with step-wise increased concentrations of paclitaxel (Taxol®) in the presence of the cyclosporin D analogue PSC 833 (2 μM), a potent modulator of the multidrug resistance phenotype. KPTA5 cells are 9-fold resistant to paclitaxel and taxotere, but do not exhibit significant resistance to vinca alkaloids, etoposide, anthracyclines, anti-metabolites, or alkylating agents. Doubling time and morphology were similar to the parental K562 cells. rt-PCR analysis revealed no alterations in the expression of the *mdr*1 and MRP genes. Cellular paclitaxel accumulation was unchanged. Cell cycle analyses showed a significantly higher proportion of K562 cells blocked in G2/M, in comparison with KPTA5 cells. In both cases, disruption of the mitotic spindles and the presence of multiple mitotic asters were comparable but occurred at lower paclitaxel concentrations in K562 cells than in KPTA5 cells. KPTA5 cells have a 2-fold increase both in 5β-tubulin mRNA expression and in the corresponding tubulin protein Class IV isotype content, as evaluated by rt-PCR and immunostaining. These cells display a novel phenotype of resistance to paclitaxel which suggests that alterations in expression of the 5β-tubulin gene may be involved in resistance to this drug.

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Introduction

Paclitaxel (Taxol®), a diterpene plant product isolated from the bark of the Pacific yew tree, *Taxus brevifolia,* has been shown to have significant activity in advanced breast and ovarian malignancies [1, 2]. Paclitaxel is a unique agent among anti-neoplastic drugs in that it promotes and stabilizes microtubule assembly, thereby altering microtubule dynamics [3, 4].

Paclitaxel is a substrate for the P-glycoprotein (P-gp) efflux pump determining the multidrug resistance (MDR) phenotype [5]. Selection of mammalian cells with paclitaxel has been shown to result both in expression of P-gp and in tubulin mutants [6]. The molecular details of microtubule alterations inducing resistance to paclitaxel remain obscure. Indeed, the occurrence of MDR mutants in these selections has complicated the analysis and made the characterization of microtubule alterations difficult. In order to identify mechanisms of drug resistance other than MDR, we have isolated drug resistant cell lines by performing selection with incremental increases in paclitaxel concentration in the presence of the MDR modulator PSC 833 [7]. We hypothesized that the presence of this potent MDR modulator would negate the selective advantage of P-gp expression, such that cells with other mechanisms of drug resistance would survive.

BODY

MATERIALS AND METHODS

Drugs and Reagents

PSC 833 was provided by Sandoz Pharmaceutical Corp. (Basel, Switzerland). Paclitaxel (Taxol®) was obtained from the Bristol Myers Squibb Company (Wallingford, CT). These were dissolved in absolute ethanol at 1 mM and stored at -20 °C. Taxotere was a gift from Rhône Poulenc Rohrer. All other chemicals and pan ß monoclonal antibody were purchased from Sigma Chemical Co. (St Louis, MO). Monoclonal antibody specific for class IV ß tubulin was purchased from Biogenex (San Ramon, CA).

Cell Culture

The human erythroleukemic cell line K562 was purchased from the American Type Culture Collection. The K562/R7 MDR cell line was derived by selecting K562 cells for resistance to doxorubicin [8]. The KPTA5 cell line was derived by exposure of the K562 cell line to increasing concentrations (0.1-4 nM) of paclitaxel in the continual presence of 2 μM PSC 833 for 18 months. The KPTA5 cell line was periodically challenged with 4 nM paclitaxel in the presence of 2 μM PSC 833. The K562/PSC cells were obtained by continuous exposure to 2 μM PSC 833 alone. All cell lines were cultured in McCoy 5A medium supplemented with 10 % newborn calf serum, 2 mM glutamine, 200 units/ml penicillin/ml and 100 μg streptomycin (Irvine Scientific, Santa Ana, CA). Cells were maintained at 37 °C in a humidified atmosphere with 5 % CO_2, and screened routinely for *Mycoplasma* by the DNA hybridization method (Gen-Probe, Inc., San Diego, CA).

Growth Inhibition Assay

Approximately 15,000 cells per well were seeded in 96-well plates and incubated with and without drugs for 72 hrs at 37°C in an atmosphere of 5% CO_2. Growth inhibition was evaluated by the MTT colorimetric method on triplicate assays as previously described [9]. The absorbance was quantitated with a ThermoMax microplate reader (Molecular Devices, Menlo Park, CA). The IC50 (drug concentration

resulting in 50% inhibition of MTT dye formation, compared to controls) was determined directly from semilogarithmic dose-response curves.

Reverse Transcriptase Polymerase Chain Reaction (rt-PCR).

Total RNA extraction and rt-PCR were performed as previously described [10]. PCR was performed in a Perkin-Elmer Cetus DNA Thermal Cycler (Norwalk, CT) using the following profile: 30 sec at 94 °C, 1 min at 55 °C (60°C for MRP), and 2 min at 72 °C. The amplimers used in this study were synthesized by Operon Technologies (Alameda, CA). Amplimers for *mdr*1 were: 3020-3037 (forward) and 3168-3187 (reverse). Amplimers for MRP were: 3069-3086 (forward) and 2522-2541 (reverse). Ribosomal cDNA (1846-1826 reverse; 1501-1520 forward) was used as an internal control.

We designed the following primers for analysis of the ß-tubulin isotypes (Arabic numerals refer to the gene, Roman numerals refer to the tubulin protein isotype class):

- M40 (class I) forward primer: 5' (-42,-22) CCA TAC ATA CCT TGA GGC GA 3'

- M40 reverse primer : 5' (226,246) GCC AAA AGG ACC TGA GCG AA 3'

- ß9 (class II) forward primer : 5' (1131,1150) CGC ATC TCC GAG CAG TTC AC 3'

- ß9 (class II) reverse primer : 5' (1301,1319) TCG CCC TCC TCC TCC TCG A 3'

- ß4 (class III) forward primer : 5' (1,15) ATG AGG GAA ATC GTG 3'

- ß4 (class III) reverse primer : 5' (223,243) AAA GGC CCC TGA GCG GAC ACT 3'

- 5ß (class IVa) forward primer: 5' (-85,-68) TCT CCG CCG CAT CTT CCA 3'

- 5ß reverse primer: 5' (167,186) TCT GGG GAC ATA ATT TCC TC 3'

- ß2 (Class IVb) forward primer: 5' (-43,-23) GTC TAC TTC CTC CTC TTC CC 3'

- ß2 reverse primer: 5' (291,300) GTT GTT CCC AGC ACC ACT CT 3'

These primers were designed using published sequence data for M40, 5ß and ß2 isotypes [11] or, in the case of ß4 isotype, a consensus forward primer and partial

sequence information generously provided by Kevin Sullivan (Scripps Research Institute, La Jolla, CA). Primers for M40, 5ß, ß2 and ß4 were designed to span introns. In the case of class II isotype, a partial nucleotide sequence of the C-terminal region was obtained by screening expressed sequence tags from the EMBL GeneBank (EST T03799), using the peptide sequence previously reported by Cowan et al. [12].

We have tested each sample over a range of different number of PCR cycles and at different concentrations of cDNA. Ribosomal cDNA was used as an internal control for standardization and comparison of samples. cDNAs were first adjusted in order to provide ribosomal PCR products which differed by less than 10%. PCR samples were analyzed by 8% polyacrylamide gel electrophoresis, stained with ethidium bromide, and analyzed by Cerenkov scintillation counting or densitometric reading of bands on an Alpha Innotech IS-1000 image analyzer (San Leandro, CA).

Evaluation of total and class IV ß tubulin isotype contents by immunoblotting.

Cells were harvested in log phase of growth and pellets were resuspended in lysis buffer containing Tris-HCl pH 6.80, $MgCl_2$ 1mM, 2 mM EGTA, and 0.2% Tween 20. Total protein was quantified by the Lowry assay and 100 µg of each sample was prepared in SDS then boiled before being applied to a 12% SDS-polyacrylamide gel. Proteins were then blotted onto a Hybond-ECL nitrocellulose membrane (Amersham, Buckinghamshire, UK) using a Sartorius apparatus (Hayward, CA). The membrane was blocked with buffer containing 5% milk and 1% bovine albumin then incubated 2 hours at room temperature with pan-ß monoclonal antibody (Sigma Immunochemicals, 1:3000 dilution) or anti-class IV ß-tubulin isotype monoclonal antibody (Biogenex, San Ramo, CA, 1:1000 dilution), washed 3 times, incubated in goat anti-mouse antibody, washed, incubated in streptavidin-biotin, washed and processed in ECL reagents. Phosphocellulose-purified tubulin prepared from bovine brain was used as a control. Preliminary experiments were performed to determine the concentration of antibodies allowing quantification of tubulin content.

Drug Accumulation Assays

Intracellular paclitaxel and vinblastine accumulation were quantitated using radiolabeled drugs as previously described [13]. Cells were incubated at 37°C in serum-free McCoy 5A media supplemented with 40 mM Hepes buffer in the presence of 50 nM [^{3}H]-paclitaxel (19 Ci/mmol, NEN-Dupont). After 2 hrs, approximately 1.5 x 10^6 cells were centrifuged through Versilube F50 silicone oil (General Electric Co. Waterford, NY) at 12,000 rpm for 1 min. The media and oil were aspirated, and the cell pellet solubilized in 4% SDS at 65 °C for 1 hr. Ecolite scintillation cocktail (ICN Biochemicals, Costa Mesa, CA) was added and radioactivity counted.

Cell cycle analysis

Cells were grown in log phase, then exposed for 24 hrs to various concentrations of paclitaxel. Cells were pelleted, fixed in ice-cold methanol for 10 min, washed, then resuspended in a solution of propidium iodide (50 μg/ml), then analyzed by fluorescent activated cell sorting. Percentages of cells in G1, S and G2/M phase were determined using CellFit® Software (Becton-Dickinson, San Jose, CA).

Immunofluorescence microscopy

Cells were seeded at densities which ensured log phase of growth throughout the duration of the experiment. Cell viability was determined by exclusion of trypan blue. Twenty-four hours after addition of paclitaxel, cells were counted to determine the increase in cell number at various concentrations of drug. Simultaneously, parallel volumes of cells were fixed for immunofluoresence microscopy.

Cells were collected by centrifugation (1,000 rpm, 3 min.) and resuspended in 5 ml of 10% formalin, 2 mM EGTA in phosphate-buffered saline (PBS) pH 7.4. Following fixation for 10 min the cells were centrifuged and resuspended in 5 ml of 99.6% methanol, 2 mM EGTA (-20 °C) for 10 min. Following three washes with PBS containing 0.1% bovine serum albumin (PBS-BSA), nonspecific antibody binding was blocked using normal goat serum (1 part serum to 4 parts PBS-BSA, 15 min). Cells were

then centrifuged and resuspended in a mouse monoclonal antibody specific for ß-tubulin (a gift from Dr. Michael Klymkowski, University of Colorado, Boulder)[14], diluted 1:500 in PBS-BSA and incubated 1 hr at 37 °C with gentle agitation. Following three washes with PBS-BSA, cells were stained with a fluorescein isothiocyanate-conjugated-goat-anti-mouse IgG (Cappel, West Chester, PA) for 1 hour at 37°C. DAPI (4,6-diamino-2-phenylindole, 1 µg/ml, Sigma) was added to the cell suspension one minute prior to centrifugation and washing (three times with PBS-BSA). Fifty µl of fixed stained cells were mounted on glass slides with approximately 30 µl of 95% glycerol containing p-phenylenediamine (1 mg/ml). Photomicrographs were obtained using a Zeiss photomicroscope III equipped with epifluorescence.

RESULTS

Resistance Profile of Paclitaxel-Selected KPTA5 Cells

The sensitivity of parental K562 and KPTA5 cells to several drugs is shown in Table 1. Compared to K562 cells, KPTA5 cells presented significant resistance (9-fold) to the selecting agent paclitaxel as well as to its analogue taxotere. No resistance, however, was observed to other tubulin interacting drugs, including the vinca alkaloids, or to various other drugs. The control cell line K562 exposed to drugs in the presence of PSC presented no significant differences in drug sensitivity compared to the parental K562 cells. Doubling time and morphology were unchanged.

Analysis of *mdr*1, *MRP* and ß-tubulin isotype expression by rt-PCR

Total RNA was extracted from the cell lines and was analyzed by rt-PCR for the presence of *mdr*1 transcripts. K562 cells and KPTA5 cells had no detectable levels of *mdr*1 transcript contrary to K562/R7, a P-gp-positive cell line which was derived from K562 and used as a control. No overexpression of the MRP gene was observed in KPTA5 compared to the parental cells.

Analysis of ß-tubulin isotype transcripts by semi-quantitative rt-PCR demonstrated identical levels of most isotypes (M40, ß9, ß4, ß2) in K562 cells and KPTA5 cells but a

consistent two-fold overexpression of the 5ß-tubulin isotype transcripts in KPTA5 cells relative to K562 cells (Table 2). This increased expression was confirmed in six different preparations.

Total tubulin and class IV tubulin content

Analysis of total ß tubulin content by immunoblotting of total cell lysates with pan-ß monoclonal antibody revealed no differences between the parental K562 and the KPTA5 variant. Identical results were obtained by fluorescence activated cell sorting of permeabilized cells with a pan-ß monoclonal antibody. However KPTA5 cells over-expressed class IV tubulin in comparison to K562 cells. The class IV monoclonal recognizes both the 5ß (Class IVa) and the ß2 (Class IVb) isotypes of tubulin, which share the same C-terminal portion of the ß-tubulin peptide chain. Although the immunostaining results cannot distinguish between these two isotypes, these data are in keeping with PCR results indicating increased expression of the 5ß isotype. The fact that total tubulin content was not altered by the increase in Class IV tubulin may be explained by the relatively low expression of this isotype, as suggested by the number of cycles of PCR required to obtain significant bands.

Intracellular Paclitaxel Concentration

The cellular accumulation of [^{3}H]-Paclitaxel was examined in parental K562, paclitaxel-selected KPTA5, and the MDR cell line K562/R7. Whereas, as expected, K562/R7 demonstrated significantly decreased accumulation of [^{3}H]-Paclitaxel, no differences were observed in [^{3}H]-Paclitaxel levels between KPTA5 and K562.

Effects of paclitaxel on mitosis, spindle morphology and nuclear morphology

In order to gain more insight into the role of altered tubulin isotype gene expression in resistance to paclitaxel, the effects of a range of paclitaxel concentrations on spindle morphology and chromatin and chromosomal organization were examined in K562 and KPTA5 cells using immunofluorescence microscopy. Paclitaxel has been found to arrest mitosis specifically at the transition from metaphase to anaphase in other cell lines (17,

M. A. Jordan, K. Wendell, W. B. Derry, and L. Wilson, unpublished results). Likewise with K562 and KPTA5 cells mitosis was blocked at this stage. Incubation of cells with increasing levels of paclitaxel for approximately one cell cycle (24 h) resulted in arrest of K562 cells in metaphase at lower paclitaxel concentrations than KPTA5 cells. K562 cells sustained complete metaphase arrest at 25 nM paclitaxel while KPTA5 cells failed to enter anaphase at taxol concentrations of 50 nM and above (Table 4). Approximately two-fold more paclitaxel was required to cause metaphase arrest in KPTA5 cells than the paclitaxel-sensitive K562 cells, with half-maximal metaphase arrest occurring at paclitaxel concentrations of approximately 30 and 50 nM for K562 and KPTA5 cells, respectively. Thus, KPTA5 cells were able to progress from metaphase to anaphase in the presence of higher paclitaxel concentrations than K562 cells.

Microtubule staining of control K562 cells and control KPTA5 cells and the corresponding DAPI-stained chromatin had similar morphology; microtubules radiated from a single centrosome, and nuclei were often multilobed. Because these cells are spherically-shaped, clear photographic images of all interphase microtubules were difficult to obtain; however, no obvious morphological distinctions were detected between either cell line in the absence of paclitaxel.

Low paclitaxel concentrations induced subtle abnormalities in mitotic spindles more frequently in the paclitaxel-sensitive K562 cells than in KPTA5 cells. For example, at 10 nM paclitaxel many K562 cells possessed bipolar metaphase spindles with a few or several misaligned chromosomes as well as multipolar spindles with scattered chromosomes. However, at this same paclitaxel concentration few KPTA5 cells had abnormal spindle microtubule morphology. Quantitation of bipolar spindles with misaligned chromosomes showed a concentration-dependent shift toward higher paclitaxel concentrations for the KPTA5 cells compared with K562 cells.

At higher paclitaxel concentrations (> 5 nM with K562 cells and >10 nM with KPTA5 cells) minor spindle abnormalities became less prominent and were replaced by

more severe multiple aster (or multipolar) spindle morphologies. For example, with 10 nM paclitaxel 55% of mitotic K562 cells possessed multipolar spindles while only 14% of mitotic KPTA5 cells contained multipolar spindles. These spindles appeared to be composed of densely packed masses of microtubules emanating out in star-shaped aggregates and the chromosomes often appeared scattered around the asters. Cells with multipolar spindles often appeared to have chromosomes aligned along several metaphase plates. Above 25 nM paclitaxel concentrations almost 100% of the mitotic cells in both cell lines were multipolar.

Interestingly, a substantial population of KPTA5 cells were multinucleate interphase cells at higher paclitaxel concentrations. The nuclear morphology of these cells was unusual in that the chromatin was often organized into several small separate nuclei rather than one large lobed nucleus. Whereas K562 cells showed only a gradual increase in multinucleation over the paclitaxel concentrations examined, more KPTA5 cells were multinucleated at lower paclitaxel concentrations. For example, at 25 nM paclitaxel, approximately 15% of K562 cells were multinucleate compared with 50% of the KPTA5 cells. Together the results suggest that KPTA5 cells with slight metaphase spindle abnormalities are able to progress through mitosis into interphase in the presence of paclitaxel to a larger degree than K562 cells; however, mitotic transit in the presence of paclitaxel results in multinucleate interphase cells.

Cell cycle analysis of K562 and KPTA5 cells exposed to paclitaxel

As shown in Table 3, in the absence of drugs K562 and KPTA5 cells had the same distribution between G1, S and G2/M phases of the cell cycle. This is in keeping with the fact that the doubling times of the two cell lines are identical. However, in the presence of 20 nM paclitaxel, there were significantly more K562 cells blocked in G2/M (28.9%) than KPTA5 cells (14.1%). At high concentrations of paclitaxel, the blockage was comparable in both cell lines.

Table 1. Drug-Resistance Phenotype in K562 and KPTA5 cell lines

	IC_{50}, nM		*(Fold resistance)*
	K562 Cells	KPTA5 Cells	
Paclitaxel	4.8 ± 1.6	43 ± 8.1	9
Taxotere	1.0 ± 0.3	9.4 ± 3.6	9
Vinblastine	4.8 ± 3.2	7.3 ± 5.3	1.5
Vincristine	1.3 ± 0.4	1.2 ± 1.2	1
Navelbine	25 ± 5.7	33 ± 11	1.3
Colchicine	10 ± 5.2	10 ± 4.6	1
Podophyllotoxin	32 ± 15	47 ± 22	1.5
Etoposide	3,250 ± 150	4,500 ± 260	1.3
Doxorubicin	12 ± 5.1	16 ± 4.2	1.3
Daunorubicin	10 ± 5.3	16 ± 12	1.6
Mitoxantrone	21 ± 16	23 ± 18	1.1
Bleomycin	26,400 ± 14,600	36,700 ± 5,800	1.4
Methotrexate	35 ± 17	36 ± 9.5	1
cis-Platinum	5,100 ± 1,800	5,300 ± 1,900	1

Concentration of anticancer drug that inhibited cell growth by 50% in a 72hr MTT assay. Values are means of at least three independent experiments ± SE

Table 2 - Expression of ß-tubulin isotypes in K562 and KPTA5.

ß-tubulin isotype	Number of cycles	KPTA5
M40	22	0.90 ± 0.20
ß9	28	1.05 ± 0.15
ß4	45	NE
5ß	40	2.30 ± 0.32
ß2	36	1.30 ± 0.25

The number of PCR cycles at which the isotype was quantified is noted in the middle column. NE: not expressed. Expression in KPTA5 cells is presented relative to expression in K562 cells. The expression of these isotypes in K562/PSC cells was identical to that observed in parental K562 cells. ß-tubulin isotype expression was analyzed by semi-quantitative rt-PCR, using ribosomal RNA as an internal control. PCR reactions were carried out at various cycles and at various cDNA concentrations to ensure that the reactions were not at saturation. Results shown indicate mean ± SE of at least 6 experiments with different batches of cells. Quantification of PCR reactions were performed by densitometric analysis of ethidium bromide stained bands.

Table 3 - Cell cycle analysis of K562 and KPTA5 cells exposed to paclitaxel

Paclitaxel nM		% G1	% S	% G2/M
0	K562	29.8	58.2	12.1
0	KPTA5	29	62	9
20	K562	30	41.4	28.6
20	KPTA5	29.6	56.2	14.1
300	K562	0.2	10.1	89.6
300	KPTA5	1.1	16.7	82.2

Cells were grown in log phase, incubated in propidium iodide and analyzed by fluorescent activated cell sorting using CellFit® software.

Table 4: Effects of paclitaxel on metaphase/anaphase ratios after 24 h incubation.

Paclitaxel (nM)	Number of Cells in Anaphase/Number of Cells in Metaphase	
	K562	KPTA5
0	0.23 ± 0.08	0.27 ± 0.04
4	0.12 ± 0.05	0.46 ± 0.20
10	0.05 ± 0.01	0.15 ± 0.04
25	0	0.08 ± 0.05
50	0	0
300	0	0

Data are the mean ± standard error of the mean of four experiments.

CONCLUSIONS

It is well established that many tumor cell mutants selected for resistance to single agents are cross-resistant to multiple unrelated chemotherapeutic agents [5]. This phenomenon, known as multidrug resistance or MDR, is associated with changes in accumulation and retention of drugs mediated by an integral membrane protein, P-gp, which is overexpressed in cells displaying the MDR phenotype [18]. Studies suggesting that the overexpression of the *mdr*1 gene occurs clinically support the hypothesis that the MDR phenotype is involved in the resistance of cancers to chemotherapy [19]. These observations have spurred clinical trials attempting to reverse MDR by combined therapy using chemosensitizers [20].

Despite the enthusiasm for MDR modulation in the clinic, other mechanisms of resistance are likely to be selected in the course of these clinical trials. The development and understanding of resistance models which arise in the setting of suppression of MDR will lead to methods for identifying such mechanisms in patients. This may in turn allow such patients to be prospectively identified, and perhaps lead to alternative and increasingly effective therapeutic strategies.

Cells possessing the MDR phenotype display cross-resistance to paclitaxel, and selection of cells with paclitaxel has resulted both in MDR and in tubulin mutants. Indeed, the predominance of P-gp positive mutants in these selections has complicated the mutant analysis and made the identification of less frequent mutations, such as those affecting microtubule function, more difficult [21, 22]. For example, Horwitz *et al.* [23] have selected an 800-fold paclitaxel resistant cell line J7/TAX50, which also display co-resistance to MDR related drugs. Modifications in tubulin expression have not been reported in these cells.

Cabral et al. were the first to suggest the use of verapamil, a chemosensitizer of P-gp, in order to select for tubulin mutants [24]. The hypothesis was that the presence of the MDR modulator would negate the selective advantage of P-gp so that cells with

other mechanisms of drug resistance would survive. They used this strategy to isolate a vinblastine-resistant mutant of CHO cells which manifested alterations in the electrophoretic mobility of tubulin suggesting mutant forms of both α and β tubulin [25]. Similarly, Fojo *et al.* have previously characterized a human breast cancer cell line (MCF-7/AdrVp100) selected in the presence of verapamil for resistance to the anthracycline adriamycin. These cells overexpressed a novel resistance related 95kDa membrane protein [26]. Ohta *et al.* have reported increased acetylation of α tubulin in H69 cells resistant to paclitaxel [27]. In the murine cell line J774.2, Haber et al. have described an increase of the Mß5 tubulin isotype associated with resistance to paclitaxel [28].

Paclitaxel (Taxol®) is a unique agent in the sense that it binds to polymerized tubulin exclusively [29] and stabilizes microtubules. It has been shown that paclitaxel induces microtubule bundling [30], and at submicromolar concentrations paclitaxel suppresses growing and shortening at the ends of microtubules in a manner similar to vinblastine [31, 32] *i.e.*, stabilizing the polymerization dynamics of spindle microtubules [17]. The binding site of paclitaxel has not been precisely determined. Rao et al., using a photoaffinity-derivative of paclitaxel, have demonstrated preferential binding to the N-terminal portion of ß-tubulin [33]. Others have shown binding both to the α and the ß subunits of tubulin [34].

In this report, we describe the characterization of a paclitaxel selected cell line, KPTA5, which was selected in the presence of increasing concentrations of paclitaxel over an 18-month period in the continual presence of the potent MDR modulator PSC 833. The KPTA5 line presents 9-fold resistance solely to the microtubule stabilizing agents paclitaxel and its analogue taxotere. However,it does not display either resistance or hypersensitivity to other drugs that act on microtubules such as vincristine or vinblastine. The cell line does not overexpress the *mdr*1 gene nor does it manifest altered accumulation of paclitaxel. Contrary to other paclitaxel-selected cell lines,

KPTA5 does not show paclitaxel-dependent cell growth or hypersensitivity to other tubulin inhibitors [35].

The most striking characteristic of KPTA5 cells is the fact that they overexpress the 5ß tubulin isotype two- to three-fold compared to the parental K562 cell line, both at the RNA and the protein level. It has previously been shown that mutagenized CHO cells selected for low paclitaxel resistance (2-3 fold) have decreased levels of tubulin isoforms and even the appearance of new ones [21, 36]. Similarly, the H69/Txl cell line which is 4.7 fold resistant to paclitaxel expresses a third isoform of α tubulin [35]. Although the involvement of the 5ß isotype in the determination of resistance to paclitaxel has not been proven in the scope of this study, it is possible that the affinity of this isotype for paclitaxel is different from that of other isotypes. If this were the case, an increased content in 5ß isotype could modify either the amount of paclitaxel which is not bound to microtubules, or the ability of paclitaxel to suppress microtubule dynamics.

Human genes coding for α- and β- tubulin constitute a multigene family of about 15 to 20 members [37]. A significant number of these, if not the majority, are pseudogenes [38, 39]. This must be kept in mind when performing rt-PCR analyses for quantification of tubulin isotypes. Ultimately, modifications observed by rt-PCR should be confirmed by analysis of the protein content of the corresponding isotype. Although there is tissue-specific expression of some of these isotypes, many analyses have detected no functional differences among isotypes (reviewed in [40]). In particular, transfection experiments have demonstrated that the exogenous isotypes are incorporated into all microtubular structures present in the transfected cells [41]. The expression of class IV isotype has been reported to be restricted, with significant expression in neuronal tissues or tumor lines [42, 12].

Tubulin synthesis appears to be tightly regulated. Cleveland et al. have described an autoregulatory mechanism taking place at the translational level, involving the first four amino acids of tubulin, which are common to all known isotypes [43]. More recent

data support the notion that adequate folding of tubulin subunits requires appropriate chaperone molecules, which act as regulators of tubulin function *in vitro* [44, 45]. Little is known concerning the regulation of the synthesis, utilization or degradation of tubulin isotypes. Of particular interest are the results reported by Sisodia et al., in which CHO cells were transfected with a chicken class IV ß tubulin construct [46]. The transfected cells actively transcribed the exogenous isotype but this protein was degraded at a high rate and did not accumulate to more than 10% of the total tubulin in the cells. Furthermore, the investigators demonstrated that transient elevation in the content of chicken class IV was met with a compensatory loss of endogenous class IV isotype. Although gene amplification is a possible mechanism of 5ß tubulin isotype overexpression in KPTA5 cells, it is more likely that translational or post-translational alterations are involved.

Microtubules are intrinsically dynamic polymers, and there is strong evidence that the dynamic properties of microtubules are critically involved in their functions [4]. Recent evidence has indicated that microtubule dynamics can be regulated by the tubulin isotype composition of microtubules [47]. In HeLa cells, low concentrations of paclitaxel appear to block mitosis at the metaphase/anaphase transition and inhibit proliferation by suppressing spindle microtubule dynamics [17]. Mitotic spindles of HeLa cells blocked in mitosis by low concentrations of paclitaxel exhibit subtle alterations in the organization of their microtubules and chromosomes. These abnormalities are believed to result from sensitive suppression of microtubule dynamics by paclitaxel [17,32].

Similar spindle abnormalities were exhibited by K562 cells and KPTA5 cells in the presence of low concentrations of paclitaxel that induced mitotic arrest. The abnormalities included formation of spindles in which a few chromosomes failed to migrate to the metaphase plate as well as formation of multipolar spindles. Significantly, KPTA5 cells required higher concentrations of paclitaxel to induce the

abnormalities than K562 cells. The induction of these abnormalities by higher concentrations of paclitaxel in KPTA5 cells is consistent with the idea that the overexpression of the β_{IV} tubulin isotype in KPTA5 cells may lead to an altered interaction of paclitaxel with the microtubules of KPTA5 cells as compared with K562 cells. Paclitaxel may bind with lower affinity to microtubules of KPTA5 cells, or it may less potently suppress the dynamics of microtubules in KPTA5 cells.

The ultimate clinical significance of the overexpression of the 5ß tubulin isotype in the determination of paclitaxel resistance will require prospective clinical investigation by analyzing ß tubulin isotype expression in human tumor samples. Better knowledge of cellular drug resistance mechanisms may assist in therapeutic strategies to circumvent such resistance. These studies should contribute to a better understanding of non P-gp-mediated mechanisms of resistance to tubulin-active agents such as paclitaxel, and open new avenues in the field of modulation of resistance to chemotherapy.

References

1. Rowinsky, E. K.; Cazenave, L. A. ; Donehower, R. C. Taxol: a novel investigational antimicrotubule agent. J. Natl. Cancer Inst. 82:1247-1259; 1990

2. Gelmon, K. A. Biweekly paclitaxel (Taxol) and cisplatin in breast and ovarian cancer. Semin. Oncol. 21:24-28; 1994

3. Manfredi, J. J. ; Horwitz, S. B. Taxol: an antimitotic agent with a new mechanism of action. Pharmacol. Ther. 25:83-125; 1984

4. Wilson, L. ; Jordan, M. A. Pharmacological probes of microtubule function. In: Hyams, J.L.; Lloyd, H.A. eds. *Microtubules* . New York; Wiley-Liss, Inc.;1994):pp. 59-83

5. Bradley, G.; Juranka, P. F. ; Ling, V. Mechanism of multidrug resistance. Biochim. Biophys. Acta. 948:87-128; 1988

6. Boggs, B. A.; Gonzalez-Garay, M. L.; O'Brien, W. E.; Barlow, S. B. ; Cabral, F. Mechanisms of cellular resistance to drugs that target microtubules. Cell. Pharmacol. 1 (Suppl 1):1993

7. Twentyman, P. R. ; Bleehen, N. M. Resistance modification by PSC-833, a novel non-immunosuppressive cyclosporin. Eur. J. Cancer. 27:1639-1642; 1991

8. Jeannesson, P.; Trentesaux, C.; Gerard, B.; Jardillier, J. C.; Ross, K. L. ; Tokes, Z. A. Induction of erythroid differentiation in human leukemic K-562 cells by membrane-directed action of adriamycin covalently bound to microspheres. Cancer Res. 50:1231-1236; 1990

9. Twentyman, P. R.; Fox, N. E. ; Rees, J. K. Chemosensitivity testing of fresh leukemia cells using the MTT colorimetric assay. Br. J. Haematol. 71:19-24; 1989

10. Brophy, N. A.; Marie, J. P.; Rojas, V. A.; Warnke, R. A.; McFall, P. J.; Smith, S. D. ; Sikic, B. I. Mdr1 gene expression in childhood acute lymphoblastic leukemias and lymphomas: a critical evaluation by four techniques. Leukemia. 8:327-335; 1994

11. Lewis, S. A.; Gilmartin, M. E.; Hall, J. L. ; Cowan, N. J. Three expressed sequences within the human beta-tubulin multigene family each define a distinct isotype. J. Mol. Biol. 182:11-20; 1985

12. Cowan, N. J.; Lewis, S. A.; Sarkar, S. ; Gu, W. Functional versatility of mammalian ß-tubulin isotypes. In: Maccioni, R.; Arechaga, J., eds. The Cytoskeleton in Cell Differentiation and Development New York: ICSU Press; 1986: pp. 157-166

13. Harker, W. G. ; Sikic, B. I. Multidrug (pleiotropic) resistance in doxorubicin-selected varian the human sarcoma cell line MES-SA. Cancer Res. 45:4091-4096; 1985

14. Chu, D. ; Klymkowsky, M. W. Experimental analysis of cytoskeleton function in early Xenopus Laevis embryos. In: Maccioni J.A. and Maccioni, R., eds. The Cytoskeleton in Differentiation and Development. Oxford: IRL Press; 1989:pp. 331-333.

15. Jordan, M. A.; Thrower, D. ; Wilson, L. Mechanism of inhibition of cell proliferation by Vinca alkaloids. Cancer Res. 51:2212-2222; 1991

16. Jordan, M. A.; Thrower, D. ; Wilson, L. Effects of vinblastine, podophyllotoxin and nocodazole on mitotic spindles. Implications for the role of microtubule dynamics in mitosis. J. Cell Sci. 102:401-416; 1992

17. Jordan, M. A.; Toso, R. J.; Thrower, D. ; Wilson, L. Mechanism of mitotic block and inhibition of cell proliferation by taxol at low concentrations. Proc. Natl. Acad. Sci. (USA). 90:9552-9556; 1993

18. Kartner, N.; Riordan, J. R. ; Ling, V. Cell surface P-glycoprotein associated with multidrug resistance in mammalian cell lines. Science. 221:1285-1288; 1983

19. Arceci, R. J. Clinical significance of P-glycoprotein in multidrug resistance malignancies. Blood. 81:2215-2222; 1993

20. Sikic, B. I. Modulation of multidrug resistance: at the threshold [editorial; comment]. J. Clin. Oncol. 11:1629-1635; 1993

21. Cabral, F.; Sobel, M. E.; Gottesman, M. M. CHO mutants resistant to colchicine, colcemid or griseofulvin have an altered beta-tubulin. Cell. 20:29-36; 1980

22. Bhalla, K.; Huang, Y.; Tang, C.; Self, S.; Ray, S.; Mahoney, M. E.; Ponnathpur, V.; Tourkina, E.; Ibrado, A. M.; Bullock, G.; Willingham, M. C. Characterization of a human myeloid leukemia cell line highly resistant to taxol. Leukemia. 8:465-475; 1994

23. Horwitz, S. B.; Liao, L. L.; Greenberger, L. ; Lothstein, M. Mode of action of taxol and characterization of multidrug-resistant cell line resistant to taxol. In: Kessel, D., eds. Resistance to Antineoplastic Drugs. Boca Raton: CRC Press; 1989:pp. 109-126.

24. Cabral, F. ; Barlow, S. B. Mechanisms by which mammalian cells acquire resistance to drugs that affect microtubule assembly. Faseb J. 3:1593-1599; 1989

25. Schibler, M. J.; Barlow, S. B. ; Cabral, F. Elimination of permeability mutants from selections for drug resistance in mammalian cells. Faseb J. 3:163-168; 1989

26. Chen, Y. N.; Mickley, L. A.; Schwartz, A. M.; Acton, E. M.; Hwang, J. L. ; Fojo, A. T. Characterization of adriamycin-resistant human breast cancer cells which display overexpression of a novel resistance-related membrane protein. J. Biol. Chem. 265:10073-10080; 1990

27. Ohta, S.; Nishio, K.; Kubota, N.; Ohmori, T.; Funayama, Y.; Ohira, T.; Nakajima, H.; Adachi, M. ; Saijo, N. Characterization of a taxol-resistant human small-cell lung cancer cell line. Jpn. J. Cancer Res. 85:290-297; 1994

28. Haber, M.; Burkhart, C. A.; Regl, D.; Madafiglio, J.; Norris, M. D. ; Horwitz, S. B. Taxol resistance in J774.2 cells is associated with altered expression of specific ß-tubulin isotypes. Proc. Am. Assoc. Cancer Res. (Toronto) 36:318; 1995.

29. Parness, J. ; Horwitz, S. B. Taxol binds to polymerized tubulin in vitro. J. Cell Biol. 91:479-487; 1981

30. Rowinsky, E. K.; Donehower, R. C.; Jones, R. J. ; Tucker, R. W. Microtubule changes and cytotoxicity in leukemic cell lines treated with taxol. Cancer Res. 48:4093-4100; 1988

31. Toso, R. J.; Jordan, M. A.; Farrell, K. W.; Matsumoto, B. ; Wilson, L. Kinetic stabilization of microtubule dynamic instability in vitro by vinblastine. Biochemistry. 32:1285-1293; 1993

32. Derry, W. B.; Wilson, L. ; Jordan, M. A. Substoichiometric binding of taxol suppresses microtubule dynamics. Biochem. 34:2203-2211; 1995

33. Rao, S.; Krauss, N. E.; Heerding, J. M.; Swindell, C. S.; Ringel, I.; Orr, G. A. ; Horwitz, S. B. 3'-(p-azidobenzamido)taxol photolabels the N-terminal 31 amino acids of beta-tubulin. J Biol Chem. 269:3132-3134; 1994

34. Combeau, C.; Commercon, A.; Mioskowski, C.; Rousseau, B.; Aubert, F. ; Goeldner, M. Predominant labeling of beta- over alpha-tubulin from porcine brain by a photoactivatable taxoid derivative. Biochemistry. 33:6676-6683; 1994

35. Ohta, S.; Nishio, K.; Ohmori, T.; Kubota, N.; Takeda, Y.; Kanzawa, F.; Takahashi, T. ; Saijo, N. Resistance to tubulin interactive agents taxol and vinca alkaloids. In: Miyazaki, T.; Takaku, F.; Sakurada, K., eds. The Mechanism and New Approach on Drug Resistance of Cancer Cells. Amsterdam: Elsevier Science; 1993:pp. 209-212.

36. Cabral, F.; Abraham, I. ; Gottesman, M. M. Isolation of a taxol-resistant Chinese hamster ovary cell mutant that has an alteration in alpha-tubulin. Proc. Natl. Acad. Sci. U. S. A. 78:4388-4391; 1981

37. Cleveland, D. W.; Lopata, M. A.; MacDonald, R. J.; Cowan, N. J.; Rutter, W. J. ; Kirschner, M. W. Number and evolutionary conservation of alpha- and beta-tubulin and cytoplasmic beta- and gamma-actin genes using specific cloned cDNA probes. Cell. 20:95-105; 1980

38. Wilde, C. D.; Crowther, C. E. ; Cowan, N. J. Diverse mechanisms in the generation of human beta-tubulin pseudogenes. Science. 217:549; 1982

39. Wilde, C. D.; Crowther, C. E.; Cripe, T. P.; Gwo-Shu Lee, M. ; Cowan, N. J. Evidence that a human beta-tubulin pseudogene is derived from its corresponding mRNA. Nature. 297:83-84; 1982

40. Luduena, R. F. Are tubulin isotypes functionally significant. Mol. Biol. Cell. 4:445-457; 1993

41. Lewis, S. A.; Gu, W. ; Cowan, N. J. Free intermingling of mammalian beta-tubulin isotypes among functionally distinct microtubules. Cell. 49:539-548; 1987

42. Lee, M. G.; Loomis, C. ; Cowan, N. J. Sequence of an expressed human beta-tubulin gene containing ten Alu family members. Nucleic Acids Res. 12:5823-5836; 1984

43. Yen, T. J.; Gay, D. A.; Pachter, J. S. ; Cleveland, D. W. Autoregulated changes in stability of polyribosome-bound beta-tubulin mRNAs are specified by the first 13 translated nucleotides. Mol. Cell Biol. 8:1224-1235; 1988

44. Gao, Y.; Vainberg, I. E.; Chow, R. L. ; Cowan, N. J. Two cofactors and cytoplasmic chaperonin are required for the folding of alpha- and beta-tubulin. Mol Cell Biol. 13:2478-2485; 1993

45. Marchesi, V. T. ; Ngo, N. In vitro assembly of multiprotein complexes containing alpha, beta, and gamma tubulin, heat shock protein HSP70, and elongation factor 1 alpha. Proc. Natl. Acad. Sci. U. S. A. 90:3028-3032; 1993

46. Sisodia, S. S.; Gay, D. A. ; Cleveland, D. W. In vivo discrimination among beta-tubulin isotypes: selective degradation of a type IV beta-tubulin isotype following overexpression in cultured animal cells. New. Biol. 2:66-76; 1990

47. Panda, D.; Miller, H. P.; Banerjee, A.; Luduena, R. F. ; Wilson, L. Microtubule dynamics in vitro are regulated by the tubulin isotype composition. Proc. Natl. Acad. Sci. U. S. A. 91:11358-11362; 1994